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EXHIBIT RAS-4

This is exhibit RAS-4 referred to in Declaration Under 37 C.F.R. 1.132 by Richard Anthony Strugnell dated

Richard Strugnell

Human antibody response to a strain-specific HIV-1 gp120 epitope associated with cell fusion inhibition

Jaap Goudsmit, Charles A.B. Boucher, Rob H. Meloen*, Leon G. Epstein†, Lia Smit, Lia van der Hoek and Margreet Bakker

PEPSCAN analysis, performed using 536 overlapping nonapeptides derived from the HTLV-III B nucleotide sequence of the region encoding the external envelope protein of 120 kDa (gp120), identified in the V3 region of gp120 a major binding site for antibodies of HIV-1-infected humans. The minimal amino acid sequence of this antibody binding site was demonstrated by multiple length scanning to be five to eight amino acids in length: (G)PGRAF(VT), i.e. amino acids 312–319. A peptide (Neu 21) containing this binding site for human antibodies (KSIRIQRGPGRAFVTIG) was synthesized and shown to induce HTLV-III B cell fusion-inhibiting antibodies in rabbits and mice. Antibodies binding to this HTLV-III B / LAV-1-specific peptide were shown to be primarily of the IgG 1 subclass, appeared within 6 months after HIV-1 antibody seroconversion in six out of 14 men studied, and persisted throughout the follow-up period of 10–24 months. The other eight seroconverting men did not develop antibodies to Neu 21 during the observation period. The appearance of antibodies to Neu 21 paralleled the capacity of the serum to inhibit HTLV-III B in cell fusion. HIV-1-infected men with Kaposi's sarcoma exhibited a similar frequency of antibodies to the synthetic peptide Neu 21 (14 out of 39, 36%) as asymptomatic HIV-1-infected men (112 out of 319, 35%). Adults with *Pneumocystis carinii* pneumonia had a significantly lower frequency (11 out of 78, 14%) of antibodies to Neu 21. Similarly, a low prevalence of antibodies to Neu 21 (8 out of 43, 19%) was observed among symptomatic HIV-1-infected children.

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Keywords: AIDS, HIV-1, neutralization, gp120, PEPSCAN.

Introduction

HIV is the cause of AIDS [1–3]. HIV-1-infected individuals with persistent HIV-1 antigenemia and a decline in antibodies to the core proteins p17 and p24 are at increased risk for developing AIDS [4–6]. In addition to these prognostic factors, studies have been performed to determine whether antibodies are produced during HIV-1 infection which may neutralize virus infectivity and possibly provide protection from the cytopathic effects of HIV-1 *in vivo*. Preliminary *in vitro* studies have indicated that higher titers of HIV-1-neutralizing and cell fusion-inhibiting antibodies were present among healthy individuals relative to those who progress to AIDS [7–9].

Entry of HIV-1 is facilitated by binding of the external envelope glycoprotein of 120 kDa (gp120) to the CD4 protein on the surface of HIV-1-susceptible cells [10–12]. Formation of syncytia by cell fusion requires cell surface expression of gp120 [13–14]. The ability of human and chimpanzee sera to inhibit infectivity of HIV-1 virions appears to correspond to the presence of cell fusion-inhibiting activity and has been associated, in part, with

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binding to domains within the carboxyl terminal half of gp120 [14–16]. Several groups have identified a variable neutralizing and cell fusion-inhibiting epitope, located between two cysteine residues at positions 296 and 331 of the HIV-1 external envelope [16–19]. The structure and location of this domain is fixed by a disulphide bond between the cysteine residues and the conserved amino acids with high β-turn potential at the top of the loop [16]. However, the antibody binding residues flanking the β-turn have been found to diverge between sequenced isolates [16–17]. This has been confirmed by the observation that experimental animals inoculated with recombinant gp120 or gp160, expressed in prokaryotic and eukaryotic vector systems, elicit neutralizing antibodies effective against only a subset of heterologous isolates [20–22]. Furthermore, antibodies from HIV-1-infected chimpanzees have been shown to bind exclusively to the amino acid sequence in the variable V3 region of the homologous viral strain in parallel with type-specific neutralization [16,25].

In the present study, PEPSCAN analysis [23,24] using 536 overlapping nonapeptides spanning the complete LAV-1/HTLV-III B external envelope was used to identify major binding sites for human antibodies to HIV-1 gp120. One such domain, corresponding to a sequence previously identified by experimental sera as a neutralizing epitope [16,25] was produced as a synthetic peptide designated Neu 21. This synthetic peptide, which induced HIV-1 cell fusion-inhibiting antibodies in rabbits and mice, was used as an antigen in an ELISA to determine the frequency of antibodies to this domain in transsectional sets of sera from HIV-1-infected homosexual men, intravenous drug users and children. Longitudinal serum specimens from individuals who seroconverted to HIV-1 were used to study the temporal development of antibodies to Neu 21 and the relationship of these antibodies to cell fusion-inhibiting activity. In addition, the subclass of Neu 21 antibodies was determined. Multiple length scanning using overlapping peptides of one to nine amino acids was used to define the minimal amino acid sequence of this antibody binding site.

Subjects and methods

Study population

Sera to be tested were obtained from four cohorts. The first group was formed by participants in a prospective study on the prevalence and incidence of HIV-1 infection and risk factors for AIDS [6]. This study was started in October 1984 and 961 asymptomatic men living in the Netherlands, who had had at least two homosexual contacts in the preceding 6 months were enrolled. Clinical data and blood samples were collected every 3 months. Of the 961 men, 723 were found to be HIV-1 antibody-negative. A total of 59 people seroconverted for HIV antibodies during follow-up and 238 persons were HIV-1 antibody-positive in the first sample taken. The last serum specimen from all seropositive individuals classified as Centers for Disease Control (CDC) groups II or III (n = 269) were used for the present study. The mean duration of follow-up was 17 months (range 2–31 months). The second group consisted of 186 patients with AIDS-Related Complex (ARC) or AIDS (CDC class IV), diagnosed at the AIDS Unit, Academic Medical Center, Amsterdam. Included were subjects from the prospective study mentioned above, who were classified as CDC IV. Serum specimens were obtained within 3 months of the diagnosis of AIDS and ARC. The third group consisted of 124 participants in a prospective study of HIV-1 infection among intravenous drug abusers. At entry 44, had antibodies to HIV-1, six developed antibodies during follow-up and 74 remained negative for HIV-1 antibodies during follow-up (mean duration 13 months, range 7–24 months) [26]. The fourth group comprised 43 children with HIV-1 infection. These children were treated at the New Jersey Children's Hospital AIDS Program. Using the new CDC classification system for children under 13 years of age, all of these children were classified as P2 (symptomatic) [27]. None of the patients received treatment with either zidovudine or interferon at the time their serum was drawn.

Antibodies to HIV-1 were determined with commercially-available enzyme immunoassays (EIA: Abbott Laboratories, North Chicago, Illinois, USA). Seropositivity was confirmed by immunoblotting [4] and a commercial recombinant EIA (Envacor, Abbott Laboratories).

PEPSCAN analysis

Overlapping peptides of HIV-1 gp120 were synthesized and tested as described previously [23,24] to determine the reactivity patterns of antisera. In short, scanning for antibody-reactive peptides (PEPSCAN) required the synthesis of every overlapping peptide in the gp120 sequence. The gp120 sequence of 545 amino acid residues can be read as 536 overlapping nonapeptides, in which peptide 1 consists of residues 1–9, peptide 2 of residues 2–10, and so on. The amino acid sequences were derived from the nucleotide sequences of the HIV-1 strain HTLV-III B (clone BH10; Los Alamos National Laboratory compilation of amino acid sequences of HIV isolates, 1987). The peptides, still coupled to solid supports, were then tested against the appropriate sera by ELISA. Absorbance values were plotted against the position of the amino-terminal amino acid of the peptide in the total sequence.

Multiple length scanning [28] was performed using the HTLV-III B sequence between the two cysteine residues at positions 296 and 331. Initially this sequence was synthesized as monomers and subsequently as overlapping dimers, trimers, tetramers, pentamers, hexamers, septamers, octamers and nonamers. These were each tested, as was carried out previously for the complete HTLV-III B amino acid sequence [23,24].

Antigens used for ELISA

Recombinant proteins

The amino terminal and carboxyl terminal portions of the HIV-1 envelope-coding sequence were produced by fusion to the 5′ coding region of the *Escherichia coli* galactokinase gene [16]. An amino terminal *env*-coding fragment extending from the KpnI to the StuI sites and comprising amino acid residues 42–204 (designated 120 N) and a carboxyl terminal *env*-coding fragment extending from the PvuII to the BglII sites and comprising the amino acid residues 288–467 (designated 120 C) were

prepared from gp120 of the HIV-1 strain HTLV-III B, clone BH10. The proteins produced were separated by preparative SDS-PAGE and purified by excision and elution.

Peptides

The peptide KSIRIQRGPGRAFVTIG (Neu 21; amino acids 305–321), was produced using the Merrifield solid phase synthesis by the European Veterinary Laboratory (EVL, Amsterdam, the Netherlands). Rabbit and mouse antisera were produced as previously described [29].

ELISA

The presence of antibodies was determined using a direct non-competitive solid phase immunoassay. Microtiter plates were coated with the recombinant proteins using 0.1 μg protein per well in 100 μl phosphate buffered saline (PBS) for 16 h at room temperature. For the synthetic peptide the protocol was modified. First wells were incubated with 0.1 mol/l NaH_2PO_4 (pH 5.0) and 0.2% volume/volume glutardialdehyde (Merck, Meppel, the Netherlands) at a volume of 100 μl per well, and incubated for 4 h at room temperature. Subsequently, the plates were rinsed three times for 10 min with 0.1 mol/l NaH_2PO_4 (pH 5.0). Then 10 ng peptide per well was incubated in 0.1 mol/l Na_2HPO_4 (pH 8.0), 100 μl/well, for 16 h at room temperature. After coating, wells were washed 10 times with PBS Tween-20 (0.1% volume/volume). Non-specific binding sites were blocked by incubating for 1 h at 37°C using 4% normal goat serum (NGS) in PBS-Tween-20. Sera to be tested were diluted 1 : 100 (unless otherwise indicated) in PBS-Tween-20, 4% NGS and incubated for 1 h at 37°C. After another washing step, horseradish peroxidase-labelled conjugates (for human sera, goat antihuman IgG, KPL, Gaithersburg, Maryland) were added, diluted 1 : 500 in PBS-Tween-20 with 4% NGS. Bound antibodies were visualized using o-phenylene diamine, the reaction was stopped with 1 N H_2SO_4. Optical density was read at an absorbance of 450 nm. Interassay variation was controlled by the inclusion of a positive human control serum in each test. For evaluation of temporal changes in antibody titer to Neu 21 in seroconverted men, a ratio was used of the test optical density (ODn) relative to the optical density of a sample 3 months prior to seroconversion (OD-3). A reaction was considered to be specific when a fourfold rise in optical density was observed relative to the sample 3 months prior to seroconversion. In the trans-sectional studies a cut-off value, based on the mean optical density of 149 HIV-1-seronegative samples plus four times the standard deviation, was used.

Subclass-specific IgG antibodies were determined using the same coating procedure as described above. Conjugate-dependent intra- and interplate variations were controlled by coating wells with IgG purified from human plasma. After incubating the peptide-coated wells with 1 : 100 serum, as previously described, and washing with PBS-Tween-20, both the peptide-coated and IgG-coated wells were incubated with monoclonal antibodies to human IgG subclasses 1–4, labelled with horseradish peroxidase (Central Laboratory of the Netherlands Blood Transfusion Service, Amsterdam, the Netherlands). Reactivity was visualized with Tetra Methyl Benzidine after washing the plates. The reaction was stopped with 4 N H_2SO_4. Optical density was read at 450 nm.

Affinity chromatography

Immuno-absorbent resins were prepared by coupling the peptide Neu 21 (about 0.5 mg) to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). One milliliter of test serum was incubated with the resin for 2.5 h at room temperature. The non-binding fraction was collected and the bound fraction was eluted with 0.1 mol/l glycine, 0.15 mol/l NaCl, dioxane (10%), pH 2.5. Binding and non-binding fractions were tested for binding to native gp160 and gp120 of HTLV-III B infected cells by radio-immunoprecipitation assay (RIPA) [30].

HIV-1 cell fusion-inhibition assay

Sup-T1 cells ($1.5–2 \times 10^5$ cells/well) were attached to 96-well microtiter plates as previously described [31]. Human B cells infected with HIV-1 strain HTLV-III B (B24-positive cells), washed twice with PBS, were added ($5–25 \times 10^5$ cells/well) in 100 μl Iscove's modified medium containing 10% fetal bovine serum and test serum diluted 1 : 20 (heat-inactivated for 30 min at 56°C). After incubation at 37° for 20 min the plates were centrifuged at a low speed for 10 min at 37°C. All experiments were performed in duplicate and each plate included positive and negative control sera. Syncytia were counted after 24 h incubation at 37°C. Serial serum specimens from 14 homosexual men, (participating in the prospective study), who seroconverted to HIV-1 were assayed for cell fusion-inhibiting activity. In the longitudinal study, the number of syncytia in the serum sample from a person 3 months before HIV-1 antibody seroconversion was considered as the patients' own negative control and called V-3. A positive cell fusion-inhibiting antibody response was defined as a Vn/V-3 value <0.25, representing a reduction in syncytia of 75%.

Results

HTLV-III B cell fusion-inhibiting activity and the recognition patterns of human sera in the PEPSCAN

Sera from two HIV-1 antibody-negative individuals did not recognize any of the synthesized nonapeptides (Table 1). In contrast, sera from HIV-1-infected individuals identified three distinct immunoreactivity peptide sequences. A reactive region of the amino acid sequence IQRGPGRAFVTIG (amino acids 309–321) bound three out of four sera tested (Fig. 1a and b). Two of these sera also identified the sequence ASDAKAYDTEV (amino acids 55–65) and one the sequence KNDTNTNSSSGRMI (amino acids 135–148). Each of these three sera had HTLV-III B cell fusion-inhibiting activity. In contrast, sera from two AIDS patients without HTLV-III B cell fusion-inhibiting activity did not recognize any of the HTLV-III B gp120 peptides tested (Fig. 1c). Similar binding specificity was observed with eight sera from HTLV-III B- or LAV-1-infected chimpanzees with HTLV-III B cell fusion-inhibiting antibodies. Each of these chimpanzee sera bound the amino acid sequence IRIQRGPGRAFVT

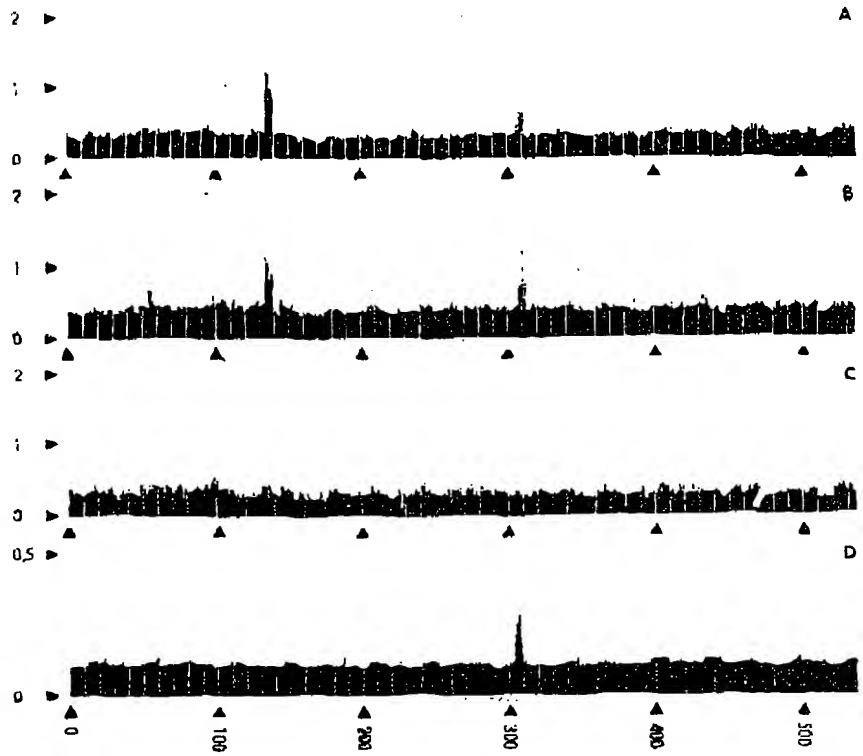

Fig. 1. PEPSCAN results using the entire amino acid sequence of HTLV-III B gp120 of three human (a–c) and one chimpanzee serum (d). Human serum was tested at a 1 : 50 dilution, chimpanzee serum at a 1 : 250 dilution. The peptides are nine amino acids long. The number on the horizontal axis corresponds to the N terminal amino acid of the nonapeptide. The amino acid sequence was derived from the nucleotide sequence of the molecular HTLV-III clone BH10. After synthesis of the peptides on a solid support, peptides were tested for reactivity by ELISA as previously described [23,24]. Prior to retesting, bound antibody was removed from the peptides. Absorbances at 450 nm obtained with each peptide were plotted vertically.

Table 1. Relationship between serum inhibition of cell fusion by HTLV-III B glycoprotein and reactivity to a linear domain in the variable V3 region of HTLV-III B gp120.

Serum source	HTLV-III B cell fusion-inhibition activity	Antibody binding to amino acids 307–321 in the V3 region
Controls		
Humans without HIV-1 antibodies	0/2	0/2
HIV-1-infected humans		
Humans without symptoms	4/4	3/4
Humans with AIDS	0/2	0/2

HTLV-III B cell fusion-inhibition tests were performed as described in the legend of Fig. 3. A titer of 1 : 20 was considered significant. The third variable region of HIV-1 gp120 (amino acids 305–330) is designated V3 according to Modrow *et al.* [34]. Antibody binding to amino acids 307–321 within V3 was determined by PEPSCAN [23,24]. PEPSCAN experiments were performed as described in the legend of Fig 1.

(amino acids 307–319; Fig. 1d). In order to confirm the relationship between cell fusion-inhibiting activity and binding to this amino acid sequence, a synthetic peptide of the HTLV-III B sequence KSIRIQRGPGRAFVTIG (amino acids 305–321) was produced, coupled to keyhole limpet hemocyanin and inoculated into rabbits and mice. These antisera inhibited HTLV-III B-induced cell fusion at a 1 : 10 titer, and immunoprecipitated HTLV-III B gp120 and its precursor protein gp160 (data not shown). Human serum with high HTLV-III B cell fusion-inhibiting activity (Fig. 1b) was fractionated by passage over a column containing immobilized peptide Neu 21. When tested in RIPA, antibodies recovered from the Neu 21 affinity resin immunoprecipitated gp120 and its precursor, gp160, of HTLV-III B-infected cells. However, no cell fusion-inhibiting activity was detected in this Neu 21 binding fraction. Multiple length scanning analysis using one human serum (Fig. 1b) and overlapping amino acid sequences between the two cysteine residues at positions 296 and 331 was performed (Fig. 2). Reactivity was first observed to a pentapeptide of the sequence PGRAF. Highest reactivity was observed to the octapeptide GPGRAFVT, defining this sequence as the crucial portion of the antibody binding site.

Temporal development of HTLV-III B cell fusion-inhibiting activity and antibodies to the V3 synthetic peptide Neu 21

Sequential serum specimens from 14 homosexual men who seroconverted to HIV-1 while under surveillance were used to study the relationship of antibodies to the V3 peptide, designated Neu 21, and *in vitro* capacity to inhibit cell fusion. A comparison was made with reactivity to the 120 N and 120 C envelope polypeptides, of which 120 C is known to induce HTLV-III B type-specific cell

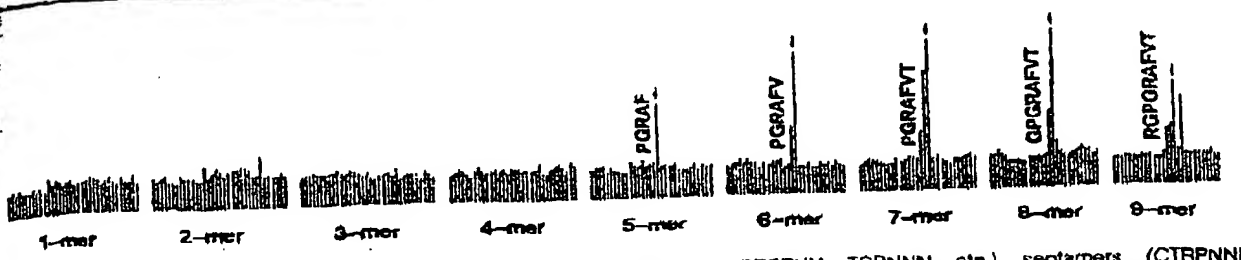

Fig. 2. Multiple length scan of serum from an HIV-1-infected man with antibodies to the V3 domain of HTLV-III B gp120. The complete amino acid sequence between the two cysteine residues at positions 296 and 331 was synthesized as monomers (C, T, etc.) and overlapping dimers (CT, TR, etc.), trimers (CTR, TRP, etc.), tetramers (CTRP, TRPN, etc.), pentamers (CTRPN, TRPNN, etc.), hexamers, CTRPNN, TRPNNN, etc.), septamers, (CTRPNNN, TRPNNNT, etc.), octamers (CTRPNNNT, TRPNNNTR, etc.), and nonamers (CTRPNNNTR, TRPNNNTRK, etc.). Subsequently, the procedure for PEPSCAN analysis was followed [23,24]. Absorbances at 450 nm obtained with each peptide were plotted vertically.

fusion-inhibiting activity in rabbits, in parallel with binding to the described synthetic peptide [16]. Sequential sera of all eight Neu 21 antibody-negative individuals lacked detectable antibodies to 120 C, and seven of these sera also lacked HTLV-III B cell fusion-inhibiting activity (Table 2). Sequential sera of five out of six Neu 21 antibody-positive individuals contained antibodies to 120 C, and five out of the six samples showed HTLV-III B cell fusion-inhibiting activity.

HTLV-III B cell fusion-inhibiting activity and antibodies to Neu 21 occurred within 3–6 months after HIV-1-antibody seroconversion (Fig. 3a), while in the Neu 21 seronegative individuals no such antibodies occurred up to 24 months after seroconversion (Fig. 3b).

Because the occurrence and disappearance of IgG subclasses is dependent on the moment of infection, we determined the IgG subclass of specific antibodies to Neu 21 in one patient (Table 2, patient 666) longitudinally. Only IgG 1 antibodies to Neu 21 were detected (Fig. 4). Subsequently, additional sera from nine individuals with Neu 21 antibodies were tested for IgGs 1–4 antibodies to Neu 21. All nine sera contained IgG 1 antibodies to Neu 21 and one also contained IgG 3 antibodies. No IgG 2 or IgG 4 antibodies to Neu 21 were detected.

Clinical disease and antibody to the V3 peptide Neu 21

Antibodies to Neu 21 were found more frequently in asymptomatic HTV-1-infected adults (112 out of 319, 35%) than in those with AIDS (39 out of 186, 21%; Table 3). This difference was statistically significant (chi-square, $P = 0.005$). No significant differences were found in seropositivity for Neu 21 between the homosexual males and intravenous drug users. Within the group of adult patients with AIDS and ARC, antibodies to Neu 21 were significantly more frequent in those patients with Kaposi's sarcoma (14 out of 39, 36%) than in those with *Pneumocystis carinii* pneumonia (11 out of 78, 14%) (chi-square, $P = 0.01$). The frequency of the occurrence of antibodies to Neu 21 in patients with Kaposi's sarcoma (36%) was comparable to that of all HIV-1-seropositive individuals without symptoms, whereas the subgroup of *Pneumocystic carinii* pneumonia patients showed the lowest percentage of Neu 21 antibodies (14%), which was significantly different from that in the asymptomatic HIV-1-infected men (chi-square, $P = 0.002$). Among children with symptomatic HIV-1 infection, 19% had antibodies to Neu 21, this was comparable to the adults with AIDS in whom 21% had Neu 21 antibodies. No significant correlation was found between the percentage of individuals seropositive to Neu 21 and the duration of

Table 2. Relationship between antibody reactivity to the V3 peptide Neu 21, the *env* polypeptides 120 C and 120 N and cell fusion-inhibition (CFI) activity.

Patient number	Antibody to			HTLV-III B CFI activity*	CDC† classification
	Neu 21	120 C	120 N		
1. Pt 1	−	−	+	−(16)	II
2. Pt 239	−	−	+	−(23)	IV-A(18), IV-C(23)
3. Pt 317	−	−	+	−(22)	IV-D(18), IV-C(22)
4. Pt 320	−	−	+	−(10)	IV-A(6)
5. Pt 342	−	−	+	−(18)	IV-A(3), IV-C(6)
6. Pt 511	−	−	+	−(16)	III
7. Pt 594	−	−	+	−(18)	III
8. Pt 1054	−	−	+	+(18)	IV-A(12)
9. Pt 16	+	+	+	+(22)	III
10. Pt 56	+	+	+	+(20)	II
11. Pt 172	+	+	+	+(24)	IV-D(26)
12. Pt 569	+	−	+	+(15)	III
13. Pt 666	+	+	+	−(18)	III
14. Pt 672	+	+	+	+(17)	III

*The latest sample included in the study, by months of follow-up since seroconversion is given in parentheses. †The interval in months from seroconversion to entry into that particular clinical category is given in parentheses. Pt, patient; CDC, Centers for Disease Control

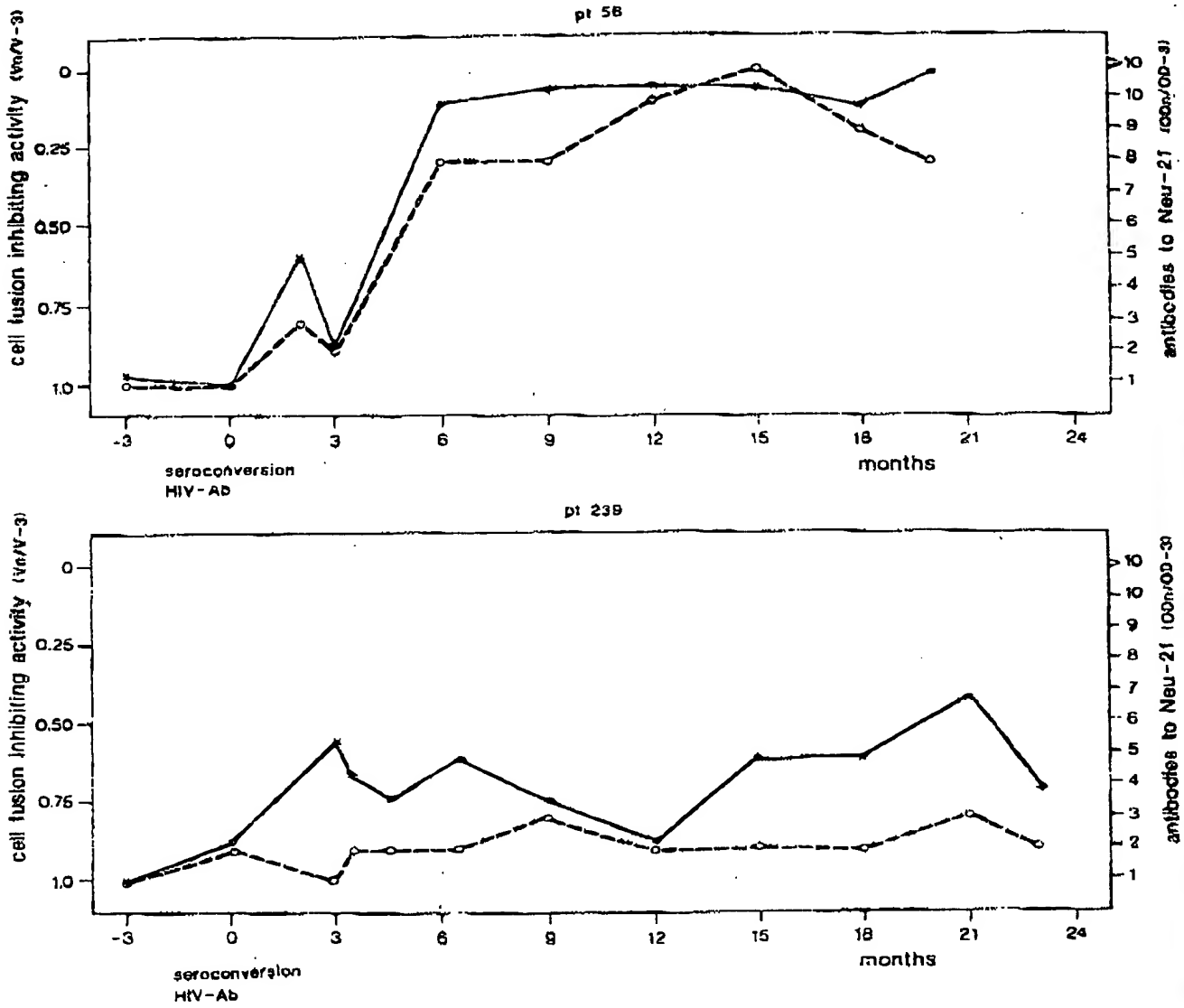

Fig. 3. Temporal development of cell fusion-inhibiting (★——★) activity and antibodies to the synthetic V3 peptide Neu 21 (O- - - -O) in patient (pt) 56 (Table 2, pt 10) having both cell fusion-inhibiting activity and antibodies to Neu 21 and in pt 239 (Table 2, pt 2) lacking both. Between 3 and 6 months after seroconversion, pt 56 showed a OD/OD-3 ratio of 8 for the Neu 21 ELISA in parallel to a Vn/V-3 ratio of >0.25 in the cell fusion-inhibiting assay. This parallel effect persisted throughout the observation period. In contrast, pt 239 never developed a ratio >4 in the peptide ELISA or cell fusion-inhibiting activity <0.25.

HIV-1 infection according to the moment of HIV-1 antibody seroconversion, confirming the all-or-nothing effect seen in sequential sera tested.

Discussion

Regions of HIV-1 that might elicit protective immunity are still undefined, although virus-neutralizing and cell fusion-inhibiting antibodies had been shown to occur at a higher titer in individuals who remain asymptomatic relative to individuals who progress to AIDS [7–9]. Antibodies produced in experimental animals by recombinant envelope proteins bound to a variable gp120 domain in parallel to type-specific neutralizing and cell fusion-inhibiting activity [16–19]. Such neutralizing and cell fusion-inhibiting activity could be abrogated by a synthetic peptide of 24 amino acids (NNTRKSIRIQRGPGRAFVCTIGKIGC) [17]. This peptide entirly blocked the cell fusion-inhibiting activity in early sera from HTLV-III B-infected chimpanzees [17]. Fine mapping identified the amino acid sequence IRIQRGPGRAFVTIG of HTLV-III B and ITKGPGRVIYA of HTLV-III RF as the binding site for strain-specific cell fusion-inhibiting antibodies in HTLV-III B/LAV-1 or HTLV-III RF-infected chimpanzees [16]. From the comparison between antibody binding patterns of HTLV-III B and HTLV-III RF cell-free virus neutralization, this domain was shown to be highly immunodominant in chimpanzees and to induce mutually exclusive antibody binding, responsible for the early type-specific neutralizing response [16,31,32].

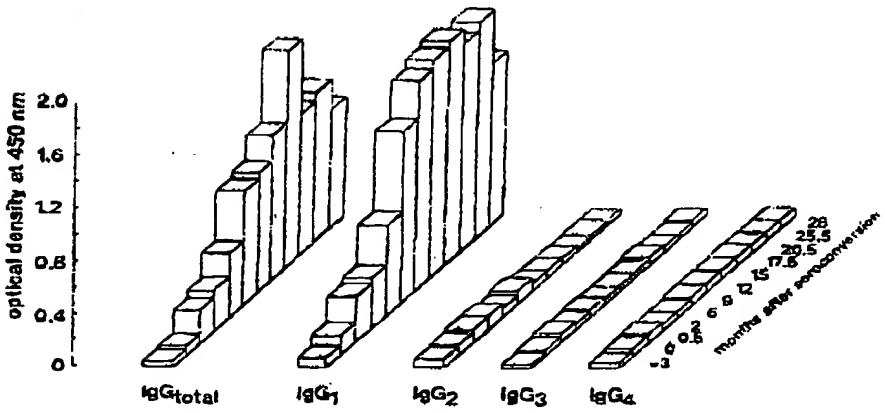

Fig. 4. IgG subclass-specific antibodies to the synthetic peptide Neu 21 in a longitudinal set of sera from an HIV-1-seroconverted man. For each sample the optical density at 450 nm is indicated in the IgG total and IgG 1, IgG 2, IgG 3, and IgG 4-specific ELISA for antibodies to Neu 21.

Table 3. Prevalence of Neu 21 antibodies in HIV-1 antibody-positive individuals in relation to AIDS-risk group and clinical disease.

Clinical category	Number tested	Number Neu 21 antibody-positive* (%)
Symptomatic (AIDS and ARC) homosexual males	186	39(21%)
Symptomatic (P2) children	43	8(19%)
Symptomless intravenous drug users	50	22(44%)
Symptomless homosexual males	269	90(33%)

*A negative cut-off optical density value was established using serum specimens from 75 HIV-1 antibody-negative homosexuals and 74 HIV-1 antibody-negative intravenous drug users. The average optical density of these specimens was 0.089. This value plus four times the standard deviation (0.055) was used to determine the cut-off optical density at 0.309.

Human sera bound strongly to one carboxyl terminal 11-amino acid sequence, RIQRGPGRAFV. A synthetic peptide encompassing this region bound human antibodies to gp120 and its precursor gp160, and by itself induced HTLV-III B cell fusion-inhibiting activity in rabbits and mice. Affinity purified human antibodies to Neu 21 did not retain cell fusion-inhibiting activity, most probably due to the purification protocol. Confirmation of the biological significance of the antigenic domain represented by peptide Neu 21 awaits the selection of human monoclonal antibodies with this peptide. The minimal sequence of this antibody binding site was demonstrated to be five to eight amino acids in length, (G)PGRAF(VT), indicating that the amino acids with high β-turn potential (GPGR) are highly conserved between sequenced isolates as structural requirements, and the variable amino acid residues at the carboxyl terminal of this fixed turn (AF) are contact residues essential for antibody binding and subsequent type-specific cell fusion inhibition. These antibodies appeared either within 6 months after seroconversion and persisted or did not appear at any time during follow-up, suggesting an all-or-nothing phenomenon. Antibodies which bound to this immunodominant epitope were primarily of the IgG 1 subclass. This confirms previous observations that reactivity to envelope antigens is primarily found in the IgG 1 antibody fraction, while reactivity to *gag* antigens occurs in all IgG subclass fractions [33]. Only limited conclusions could be reached about the clinical significance of these antibodies, due to the variable nature of this antibody binding site. However, AIDS patients, particularly those with opportunistic infections, were less frequently found to have antibodies to Neu 21 compared to asymptomatic HIV-1-infected individuals. Patients with Kaposi's sarcoma exhibited the same frequency of antibodies to Neu 21 (36%) as asymptomatic HIV-1-infected individuals (36%). The all-or-nothing response to this domain indicates the type-specific nature of these neutralizing antibodies. The decreased frequency of antibodies to Neu 21 with the advance of the disease may indicate a role of this domain in protection against disease progression. This could be important for a therapeutic strategy utilizing post-infection immunization, especially because the location and structure of this variable region is highly conserved. Firm conclusions, however, await the results of seroepidemiological studies using peptide antigens encompassing the conserved β-turn and divergent contact residues in conjunction with sequence analysis of this region from the patient's own virus strain(s).

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